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The role of Integrins and IGFBPs in the IGF-I Stimulated Migration of Human Breast Cancer Cells

Task 1 Months 1-6: Complete migration assays with MDA-231 and MCF-7 cells with laminin, collagen (types I and IV) and fibronectin using IGF-I, EGF, FGF, PDGF and hepatocyte growth factor. Compare the response to des 1-3 IGF-I and IGF-II to IGF-I. Continue work to complete the analysis of the response to EGF, FGF and PDGF using each of the 4 matrices. This should be completed by May 1998.

Task 2 Months 5-16: Perform immunoprecipitation of the integrin studies on four additional candidate cell lines, then conduct assays to determine:

- a. whether IGF-I stimulates their migration and compared IGF-I's effects to those of other growth factors.
- b. whether the increased migration, if present, is due to chemotaxis
- c. study the effects of anti-integrin antibodies, using the pattern of integrin expression for each cell as a guide

This work is progressing well at present. The integrins that are present in the four additional cell lines have been defined. This should be completed by September, 1998.

Task 3 Months 6-24: Study the calf serum effect on MCF-7 cells in greater detail by performing:

- a. cross-linking studies for IGF-I receptors.
- b. migration assays with cells grown in calf serum with supplemental IGF-I.
- c. migration assays with cell growth in media containing tamoxifen and other anti-estrogens.
- d. ligand blots and immunoblots on conditioned media from cells with various calf serum exposure times to determine any changes in IGFBP expression.

Task 4 Months 16-36: Determine the effects of IGFBP-1 through BP-5 on IGF-I stimulated migration:

- a. add each IGFBP to the upper and lower chambers immediately prior to migration assays.
- b. perform migration assays with several different breast cancer cell lines grown in media containing a supplemental amount of each IGFBP
- c. use IGF-I analogs with decreased IGFBP affinities to study IGF-I independent effects

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INTRODUCTION

Insulin-like growth factor-I (IGF-I, formerly known as somatomedin C) is one of the broad spectrum polypeptide growth factors with biological actions in diverse tissues (1). The amino acid sequence of IGF-I has 48% homology with human proinsulin (2). When IGF-I binds to its receptor, the 185 kilodalton (kDa) protein known as insulin substrate-1 (IRS-1) undergoes tyrosine phosphorylation. IRS-1 mediates cellular responses to stimulation of both IGF-I and insulin receptors (3). A study published in 1984 first established that IGF-I stimulated the growth of human breast cancer (HBC) cells kept in long-term tissue culture (4) and physiologic levels of IGF-I stimulate DNA synthesis in the absence of other serum components. This study also showed that the HBC cell membranes have IGF-I receptors (4). Later studies performed on breast cancer biopsy specimens evaluated IGF-I receptors and found that the cancerous tissue had higher levels of this receptor than did adjacent normal tissue from the same specimen (5).

Stewart et al. (6) discovered that estradiol sensitizes MCF-7 cells to the mitogenic effects of IGF-I by increasing mRNA for the IGF-I receptor. Subsequent studies (7-9) have examined the effects of α IR-3, a murine monoclonal antibody to the IGF-I receptor. This antibody significantly limited growth of both MCF-7 and MDA-231 cells *in vitro* (8).

Several authors have reported that IGF-I can stimulate migration of malignant cells, including human melanoma (10) cells and pancreatic carcinoma (11) cells. These experiments and others utilized a modified microwell Boyden chamber. Because it is necessary in the early stages of the metastatic process for tumor cells to bind to and migrate through basement membranes to gain access to the circulatory system, use of the Boyden chamber allows for study of the invasive capacity of cancer cells.

Others have studied malignant breast cells in Boyden chamber assays. Verhasselt and colleagues (12) found that interleukin-1 and interleukin-6 stimulate migration of SK-BR-3 and ZR-75-1 cells. Hansen et al. (13) studied the effects of $1\alpha,25$ dihydroxyvitamin D₃ on MDA-231 cells. Tehy found that after a seven day exposure to $1\alpha,25(\text{OH})_2\text{D}_3$, the invasive capacity of these cells was markedly reduced. Interpretation of Hansen's results is complicated by the fact that her group used Matrigel™, which contains IGF-I at a 15 ng/ml concentration to coat their membranes.

Given the proven mitogenic effects of IGF-I on HBC cells and its ability to induce migration in other malignant cell types, our work began with the hypothesis that IGF-I would stimulate migration of breast carcinoma cells. We selected MCF-7 and MDA-231 as representative of ER positive and ER negative breast cancer cells, respectively. Our initial studies were published in February of 1996 in the Journal of Biological Chemistry (JBC) (14). We found that IGF-I stimulated migration in both cell lines. The conditions required for the MCF-7 cells to migrate in response to IGF-I were much more specific than those for the MDA-231 cells. The MCF-7 cells would only migrate through type IV collagen or vitronectin-coated membranes. (Fig. 1, ref. 14). Interestingly, they do not migrate in response to their growth media, which contains 10% fetal calf-serum.

Once a migratory response to IGF-I had been established, we conducted checkerboard assays to assess the relative amount of chemotaxis (directional migration) versus that of chemokinesis (random movement) caused by IGF-I. These studies (Fig. 4, ref. 14) revealed that the majority of increased cellular migration was, in fact, directional towards IGF-I. Marked inhibition of IGF-I-stimulated migration was seen in both cell lines when they were pre-

incubated with an anti-IGF-I receptor antibody (termed α IR3), confirming the necessity of normal receptor function. Additional studies were done to compare IGF-I's effect with that of other known and likely chemoattractants. The MDA-231 cells demonstrated a similar degree of stimulation by epidermal growth factor (EDF), but the effects of platelet deprived growth factor (PDGF-BB) and fibroblast growth factor (FGF) were not as pronounced (Fig. 3, ref. 14). In contrast, the MCF-7 cells had essentially no response to any other chemoattractant, regardless of the membrane coating substance used.

Within the past few years, the role of integrins in cellular motility and invasion has been recognized. The integrins are a superfamily of heterodimeric cell-surface glycoprotein receptors composed of distinct α and β subunits. Adhesion of cells to their extracellular matrix (ECM) is primarily mediated by these receptors; furthermore, integrins have a central role in transducing signals from the extracellular environment. Immunohistochemical studies have demonstrated that breast carcinoma tissue has altered integrin expression compared to normal breast epithelium (15). Coopman et al. (16) examined the effects of various basement membrane (BM) components on directional migration of MCF cells on glass coverslips. They found that laminin arrested migration of one MCF-7 variant while collagen type IV and fibronectin had no effect. A follow-up study by the same group (17) found no involvement of a 67 kD high-affinity laminin receptor (which is not an integrin) in this phenomenon. Since the MCF-7 cells express a variety of integrins capable of binding laminin, this study raised the possibility that these receptors mediate this effect. Using a hydrated collagen gel containing heterotypic cell populations including MCF-7 cells, Rossi et al. (18) found that the presence of fibroblasts was necessary for MCF-7 cells to migrate. It has been shown that fibroblasts secrete IGF-I (19) therefore; this requirement of MCF-7 cells may actually reflect the cells' need for IGF-I in the setting of migration. Using a different cell line, 8701-BC (isolated from a highly invasive ductal infiltrating carcinoma), Luparello and colleagues (20) found that type I-trimer collagen had a permissive effect on cell locomotion.

Two papers published in 1994 established links between vitronectin receptors and growth factor signaling. Vuori and Rusolahti (21) reported that stimulation of rat fibroblasts with insulin led to association of the α V β 3 integrin (a vitronectin receptor) with IRS-I. As discussed earlier, IRS-1 is an intracellular protein which mediates signaling by both insulin and IGF-I. This publication also utilized FG human pancreatic carcinoma cells which do not normally express the alpha V beta 3 (α V β 3) integrin and found that transfecting the FG cells with β 3 integrin complementary DNA increased their response to insulin. Specifically, the authors stated that the transfected cells expressing α V β 3 integrin responded to insulin with a 2.5-fold higher level of DNA synthesis when grown on vitronectin compared to collagen. Cells without the α V β 3 integrin had the same response to insulin regardless of the plating substance used. The doses of insulin used in these studies (up to 100 nM) are sufficient to cause stimulation of IGF-I receptors. The relative contribution of insulin receptors versus IGF-I receptors was not investigated.

A second study by Klemke et. al (11) also using the FG cells found that the epidermal growth factor (EGF), was necessary for these cells to migrate on vitronectin. The FG cells utilize the α V β 5 integrin for attachment to vitronectin, and this process does not require EGF. However, tyrosine kinase signaling by the EGF receptor was required for their migration on vitronectin but not on collagen. These cells display constitutive migration on collagen which is not affected by the EGF receptor specific tyrosine kinase inhibitor tyrphostin 25. This same substance prevents EGF-induced FG cell migration on vitronectin. As discussed below, this association between a growth factor and integrin signaling as a prerequisite for migration is

almost certainly present with MCF-7 cells as well. Our laboratory performed immunoprecipitation studies to characterize the pattern of integrin expression in the MDA-231 and MCF-7 cells (Fig. 2, ref 14). Numerous migration assays done with anti-integrin antibodies have confirmed that integrins play a key role in IGF-I stimulated migration. Furthermore, the effects of anti-integrin antibodies are highly specific for the coating substance used and vary with the relative amounts of integrins expressed by a given cell line (Fig. 6, ref. 14).

Included in our original grant proposal was preliminary data related to the effect of calf serum exposure on MCF-7 cells. We found that MCF-7 cells maintained in 10% fetal calf serum (FCS), lost their capacity to migrate in response to IGF-I. Remarkably, the same MCF-7 cells, which had shown no migration, could be grown under identical conditions and split into two different plates--one containing 10% fetal calf serum and the other containing 10% calf serum. Ten days later, typically after two more passages, the cells in the calf serum would migrate well when stimulated with IGF-I while the group of cells continued in fetal calf serum would not migrate in any measurable numbers. No such loss of migratory ability was observed in the MDA-231 cells maintained for several months in 10% FCS. To determine the time period of exposure to calf serum that was necessary for cellular migration, an assay was done with seven separate populations of MCF-7 cells grown under different circumstances--six populations had been exposed to calf serum for varying periods of time and a control population which had always been in fetal calf serum. This assay revealed that exposure to calf serum for seven to ten days was associated with the most active migration. Interestingly, cells which had been in calf serum for over a month demonstrated nearly the same poor migratory response to IGF-I as cells which had always been maintained in fetal calf serum. As part of the work proposed in our grant, we performed cross-linking studies on MCF-7 cells grown in both 10% FCS and 10% calf serum.

The biologic activities of IGF-I are largely regulated by a group of high affinity IGF binding proteins (IGFBPs), six of which have been cloned and sequenced. These IGFBPs have negligible affinity for insulin and are present in the circulation and throughout the extracellular space. They are also found in the conditioned media and extracellular matrix of most cells in culture. These binding proteins have at least four major functions, which include acting as transport proteins and prolonging the half-life of IGF-I. In addition, they provide a means for IGF localization to specific tissues and cell types and directly modulate the interactions of IGF with its receptor (I). The role of these IGFBPs in HBC cell growth and metabolism has been an area of intense investigation in the past several years.

One published study reported a correlation between ER status and IGFBP secretion in breast cancer cell lines. It showed that ER negative cells secrete IGFBP-1 and IGFBP-3. In contrast, the ER positive lines were found to secrete IGFBP-2 and IGFBP-4 (22). Subsequent studies sought to understand how IGFBPs affect the growth of HBC cells. One study demonstrated that IGF-I increased cell numbers of sparse MDA-231 cultures by 24% after 48 hours (23). Addition of IGFBP-1 led to a 45% increase while IGFBP-2 had no potentiating effect. However, IGFBP-1 in the absence of IGF-I had no effect on cell growth. A different group examined the actions of IGF-I and IGFBP-1 on MCF-7 cells. The MCF-7 cells normally require estrogen for growth; nevertheless, IGF-I can substitute for estrogen in short-term studies. In contrast to the findings for MDA-231 cells, IGFBP-1 was shown to block IGF-I induced mitogenesis in MCF-7 cells (24). After the identification of a sixth IGFBP, Sheikh et al. (25) examined four ER positive and three ER negative HBC cell lines for expression of IGFBP-5 and IGFBP-6. All cell lines except the MDA-231 expressed IGFBP-5 mRNA. In 1994 Chen et al. (26) reported that effects of multiple IGFBPs on IGF-stimulated DNA synthesis in MCF-7 cells.

They found that IGFBP-2 and IGFBP-3 enhanced DNA synthesis in response to IGF-I while IGFBP-4 and IGFBP-5 had no effect.

The expression of IGFBPs has also been examined in breast cancer tissues, where the correlation with ER status is not as clear-cut. Pekonen et al. (27) found that only IGFBP-3 expression could be correlated to ER status, being more common in ER negative tumors. They discovered that each of the tissue specimens, regardless of ER status, expressed all five of the IGFBPs that were known to exist at the time of publication. A more recent 1994 study by McGuire et al. (28) performed ligand blots for IGFBPs on eighty breast cancer tissue specimens. They were unable to detect IGFBP-1 in any of the specimens. A positive correlation of ER expression and IGFBP-4 expression was found, while IGFBP-3 was negatively correlated with ER status.

Two of the IGFBPs have been shown to have direct effects independent of IGF-I. In 1989, Blat et al. (29) reported that IGFBP-3 (then known as inhibitory diffusible factor 45) can inhibit serum stimulation of DNA synthesis in the chick embryo fibroblast cell line. Later, Jones et al. (30) found that IGFBP-1 stimulates migration of the Chinese hamster ovary (CHO) cells in woundings assays even in the absence of IGF-I. A single amino acid substitution in the IGFBP-1 sequence which prevented its binding to the $\alpha 5 \beta 1$ integrin on the CHO cell surface reversed this stimulation. Oh and co workers (31) reported that IGFBP-3 has IGF-receptor independent activities in an ER negative cell line, Hs578T. These authors found that treatment of the Hs578T cells with exogenous IGFBP-3 significantly inhibited their monolayer growth. This inhibitory effect could be overcome by native IGF-I, but not by IGF analogs with normal affinity for the IGF-I receptor but decreased affinity for IGFBP-3. This finding suggested that the effect was independent of the IGF-I receptor but was blocked by the IGF-IGFBP-3 interaction (20).

BODY

METHODS

Migration Assays - The Boyden chamber migration assays were performed by filling the lower wells with a putative chemoattractant or control solution, over which the coated polycarbonate membrane (pore size-8 μm) is placed. Phenol-red free DMEM/F12 with 0.01% bovine serum albumin (BSA) is used as a negative control solution, and all growth factors are prepared in this solution. Monolayers of confluent or near confluent cells are trypsinized and re-plated twelve to sixteen hours prior to each assay. Each assay begins by rinsing the cells with PBS/EDTA (phosphate buffered saline containing a 5 mM concentration of EDTA) and separating them from the tissue culture dish with 2 ml of 0.1X trypsin in PBS/EDTA. Then 8 ml of a 10% serum containing media are added to inactivate the trypsin. The cell suspension is centrifuged once for 10 minutes; the serum containing media is then removed. Next the cells are resuspended and washed with the DMEM/BSA solution twice. After the second centrifugation, the supernatant is removed. The cell pellet is then re-suspended in a final volume of one to three ml. A hemacytometer is used to estimate the density of the cell suspension.

For those experiments in which an anti-integrin antibody is used, the cells are separated into microcentrifuge tubes and incubated with the antibody by gently tumbling at room temperature for thirty to sixty minutes. All anti-integrin antibodies were used at a concentration of 25 $\mu\text{g}/\text{ml}$. For those experiments in which IGFBP-1 or its [221 Tryp] mutant were used, the BP-1 (final concentration 10 nM or 50 nM) was added to the cell suspension. The suspension was then tumbled at room temperature for 45-60 minutes. A 50 μl volume of the suspension containing approximately 10,000-15,000 cells (MDA-231) or 50,000-60,000 cells (MCF-7) is loaded into each upper well, and the chamber is placed in a 37°C 5%CO₂-enriched incubator. After a four hour period, the chamber is disassembled. The cells adherent to the upper surface of the membrane are scraped away so that only those cells which have migrated through the membrane remain. The membrane is then fixed in methanol, stained with Diff-Quik and allowed to air dry on a glass slide. A grid eyepiece in our microscope is then used to reliably demarcate several areas within each individual well in order to count migrated cells. Two full grids are counted for each of the MDA-231 cell wells and four grids are counted for each MCF-7 well.

To assess the ability of these cells to respond to a chemokinetic stimulus as opposed to a chemotactic stimulus, cultures were grown to confluency in 6 well plates. Both MCF-7 and MDA-231 cells were tested in this assay. The cultures were then wounded with a razor blade. Wounds had to be clean in that no cells could be attached across wound margin and no grooves could be cut in the plate, since the cells tend to migrate along the grooves. Following wounding, the wounds were scored by a blinded observer. Then the treatments, e.g. IGF-I or no IGF-I, were added at a concentration of 100 ng/ml in 0.2% calf serum. After 48 hours, the number of cells crossing the wound margin by at least 150 microns was determined. These cells were fixed to the plate by adding 70% methanol then stained with methylene blue and the number of cells migrating at least this far in each wound margin was determined.

Cross-linking Studies - Two populations of MCF-7 cells were studied, those which have been exposed to calf serum (CS) for 7-10 days and those which have always been in fetal calf serum (FCS). Both groups were trypsinized and replated in the appropriate medium in a six well plate twelve to sixteen hours prior to cross-linking. This sequence of events reproduced the conditions under which MCF-7 cells migrate well to IGF-I. The cells were rinsed twice with binding buffer (HCO₃-free EMEM with 0.1% BSA and 20 mM Hepes at pH 7.3), then

incubated with 3 to 10 μ Ci of 125 I-IGF-I with increasing concentrations of unlabelled IGF-I. Following a 90 min incubation at 4°C, the wells were aspirated and rinsed twice with cross-linking buffer, which contained EMEM without amino acid with 20 mM Hepes at pH 7.3. Immediately prior to cross-linking, freshly prepared 10 mM DSS in DMSO was added to the cross-linking buffer to yield a final concentration of 0.5 mM. The cells were then incubated in the DSS in cross-linking buffer for 30 min at 4°C. The DSS was then aspirated and neutralized with 0.1 M Tris-HCl with 0.1M NaCl at pH 7.4. The wells were rinsed twice with this neutralizing buffer, and received a third rinse of 5 to 10 min duration. The buffer was aspirated and the cells were lysed in minimal volumes SDS sample buffer with 100mM DTT (dithiothreitol) to achieve reducing conditions. Standardized samples were resolved in a 6% SDS-PAGE.

Integrin Receptor Immunoprecipitation - To determine the presence of various integrins on MCF-7 or MDA-231 cells, the total cell surface proteins were labelled by adding 1 mCi of 125 I to confluent cultures in three 10 cm dishes. Chloramine T was then added and the reaction stopped by the addition of sodium metabisulfite. The iodinated proteins were harvested by adding 1 ml of RIPA buffer. Membranes were then centrifuged and the non-membranous material discarded. The radiolabelled proteins in the membranes were solubilized by adding 100 μ l of laemmli buffer to each test sample then centrifuged in a microfuge for 10 minutes. After boiling, the supernatant was then analyzed by SDS-PAGE followed by direct autoradiography. To determine if particular integrins reacted with specific antibodies, prior to electrophoresis the RIPA lysate was incubated with a 1:500 dilution of various anti-integrin antibodies and then the complexes immunoprecipitated by the addition of protein A sepharose. The pellets were solubilized in 100 μ l of laemmli sample buffer, and 60 μ l of this mixture was loaded on SDS-PAGE gel 12.5% gel. Following electrophoresis, the gel was dried and a direct autoradiograph obtained to determine radiolabelled band intensities at various molecular weights. Correct standards were run in a parallel lane. SDS sample buffer contained dithiothreitol.

RESULTS

Task 1 - Task 1 has been completed for IGF-I, IGF-II, and des IGF-I for each of the four matrices that were listed. During the past 4 months, we have completed the studies with laminen, types I and IV collagen, and fibronectin, assessing the responses to EGF, FGF, and PDGF using each of the four matrices with both cell lines. The results are shown in Table I. EGF did not stimulate the migration of either cell type on any of the four matrices tested. FGF stimulated some enhancement of migration on fibronectin matrix. However, it had no effect on laminen, type I or type IV collagen. PDGF was a potent stimulant of migration in both cell lines on both fibronectin and type IV collagen. It had minimal effects on type I collagen and no effect on laminen. In summary, laminen appeared to inhibit the migration response to PDGF or FGF of both cell lines. The MDA-231 cells appeared selectively responsive to FGF on certain specific matrices, but not on others. No cell line appeared responsive to EGF. This work completes these studies as listed in the revised statement of work for task 1.

Task 2 - We have completely defined the integrins that are present on the 4 additional cell lines, that is in addition to MCF-7 and MDA-231. These are listed in the accompanying table III. We have assessed the response of each of the 4 additional cell lines to IGF-I in terms of cell migration and determined whether the response is due to chemotaxis. These results are shown in Tables II and III.

IGF-I stimulated the chemokinetic response of all four cell lines potently, as shown in Table II. The response to IGF-I was significant at all concentrations tested, and it was extremely potent, particularly in the ZR-75-1 line. Based on these results, we assessed its chemotactic activity, Table III. Again, IGF-I was a potent chemotactic stimulant. Therefore, clearly the migration effects are due to stimulation of chemotaxis as well as chemokinesis. Although there were differences in the responsiveness of the cell lines (e.g. ZR-75-1 responded better than BT20), all responded to IGF-I. We are currently completing the work comparing the responses to other growth factors, such as PDGF, FGF, and EGF, and, as stated in our previous revised statement of work, the studies should be completed in September 1998. We have also analyzed the effect of some of the anti-integrin antibodies (data shown in Table IV). The results show that, in general, where $\beta 3$ or $\alpha 5$ subunits are present, anti-integrin antibodies strongly inhibit migration. The response to $\alpha 3$ was more variable, being potent in BT20 cells but less potent in HS578T cells and ZR-75-1 cells. Anti- αV was a potent inhibitor of migration in HS578T cells. Other antibodies still to be tested include $\beta 1$ and $\alpha 2$. Likewise, not all of the antibodies have been tested against MCF-7 and MDA-231 cells, although some had been previously reported in last year's summary. These studies should be completed in the next 2-3 months.

Task 3 - The cross-linking studies in task 3A and migration assays in 3B have been reported previously. Prior to conducting the studies in 3C, we thought it would be helpful to determine the response of estrogen receptor positive cell lines, such as MCF-7 cells, to estradiol themselves in order to properly interpret the response to anti-estrogens such as tamoxifen. To that end, we have assessed the chemokinetic and chemotactic response of MCF-7 cells to estradiol in the presence or absence of IGF-I. The results are shown in Table V. As reported previously, the cells were very responsive to IGF-I. However, interestingly, it has not been reported previously that estradiol also stimulated their migration, but to a lesser extent. The combination of IGF-I plus estradiol was additive at 10^{-6} M estradiol. These findings suggest that tamoxifen may have an inhibitory effect, and this result will be further analyzed in the third year of the grant application. The studies in task 3D have been completed. We have performed ligand and immunoblotting of conditioned media from MCF-7 cells at various stages of exposure to calf serum. The results show definitively that the major binding proteins produced by MCF-7 cells are IGFBP-2 and IGFBP-4. The abundance of these proteins is reduced by exposure to calf serum as compared to fetal calf serum, particularly that of IGFBP-2. IGFBP-3 appears to be by far the most sensitive. That is, when these cells are grown in fetal calf serum, there is abundant IGFBP-3 produced, but when they are switched to calf serum, this production is reduced by over 90% after 12 hours of exposure. The changes in IGFBP-2 and IGFBP-4 are slower, with approximately 50% reduction of IGFBP-2 after 24 hours and an 85% reduction after 72 hours. Therefore, the change in IGFBP-3 is sudden and persistent, whereas the change in IGFBP-2 requires longer exposure times, as does IGFBP-4. This probably accounts for the enhanced sensitivity of these cells to IGF-I after calf serum exposure.

Task 4 - In year one, we completed the studies measuring the cell migration and response of MDA-231 cells to IGFBP-1. We have now completed the studies of the response of this cell line to IGFBP-3 and -5. The results in Table VI show that neither IGFBP-3 or -5 has IGF-independent effects on cell migration. That is, they do not alter cell migration in the absence of IGF-I. In contrast, the addition of IGFBP-3 inhibits the cell migration response to IGF-I, and the addition of IGFBP-5 augments cell response to IGF-I. The effect of IGFBP-5

is particularly striking, resulting in a 72% increase in cell migratory responsiveness to IGF-I. This may account for the reduced response to des IGF-I by this cell line reported previously as compared to native IGF-I. During the coming year, we plan to repeat these studies using MCF-7 cells. We will also repeat the Boyden chamber studies after growing the cells in defined amounts of IGFBPs for several days prior to analysis, and we will repeat the migration studies in the presence of IGFBP-2, since it is also one of the main proteins produced by these cells.

DISCUSSION

These results show some striking changes in the response of breast cell lines to changes in extracellular matrix, integrin display, IGF-I, and estradiol. The results reinforce the previous conclusion that many of the growth factors, such as EGF and FGF, are poor stimulants of breast cell migration, but PDGF and hepatocyte growth factor appear equally potent for some cell lines as compared to IGF-I. Clearly, changing extracellular matrices made a major difference in IGF-I and/or other growth factor responsiveness. As for many cell types, laminin is a relatively strong inhibitor of migration, as is type I collagen, whereas type IV collagen has intermediary activity and fibronectin appears to be a potent stimulant of migration. An interesting extension of these studies would be to determine if matrices are changing responsiveness to growth factor by direct interaction between the integrin mediating signaling pathways and the growth factor signal transduction pathways.

The chemokinetic data analyzed in this series of experiments show that this is also a valid assay for measuring cell migration responses to IGF-I, although not as specific as chemotaxis. Clearly, however, this assay is sensitive and represents an excellent screening tool for looking for compounds that may be active in the chemotactic assay. This assay was useful in screening multiple other cell lines for IGF-I responsiveness, and their responses in this assay correlated with the chemotactic responses of similar cell lines. Using the Boyden chamber assay, we were able to show that several anti-integrin antibodies inhibited the migration of these other cell lines in response to IGF-I. Clearly, the migration of each of these cell lines appears to be somewhat dependent upon the integrin display. While these results are incomplete at present, they suggest that the $\beta 3$ and $\alpha 5$ integrins are potent mediators of migration in several of the cell lines; whereas the display and response to αV and $\alpha 3$ is more restricted. Continuing this work and definitively determining which integrins are involved will be an important first step in extending these studies to examine signalling pathway cross-talk, as previously suggested.

These studies for the first time define the role of estradiol in mediating cell migration in MCF-7 cells. Given a stimulatory role for estradiol, it will be very important to determine whether or not tamoxifen results in inhibitory effects and whether these effects are specific for IGF-I-mediated stimulation of migration. The role of IGF binding proteins in controlling IGF-I action was extended by these studies. Specifically, IGFBP-3 and -5 were examined. Clearly, these binding proteins appear to have different effects on migration, and part of the enhanced MCF-7 response to IGF-I after growth in calf serum may be due to attenuation of IGFBP-3, since this was found to be a potent inhibitor to migration in this cell line. In contrast, IGFBP-5, which is sequestered in the extracellular matrix, was a potent stimulant of migration. This suggests that IGFBP-5 sequestration in extracellular matrix may be important in this system. This question can be further analyzed by using mutants that we have prepared that bind poorly to extracellular matrix to determine their role in modulating IGF-I-mediated cell migration in these cell lines.

CONCLUSIONS

1. Changing extracellular matrix proteins alters the cell migration response to PDGF and FGF.
2. Four additional breast tumor cell lines, ZR-75-1, BT-20, T47D, and HS578T, respond to IGF-I with increases in migration.
3. The four cell lines possess different integrins and respond differently to IGF-I in the presence of anti-integrin antibodies. The $\alpha 5$ and $\beta 3$ integrin subunits appear to be the most important for IGF-I responsiveness in several cell lines.
4. MCF-7 cells respond to estradiol with increased migration, and it enhances their response to IGF-I.
5. IGFBP-3 and IGFBP-5 modulate the migration response to IGF-I, and these two forms of IGFBPs have differential effects.

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TABLE I

Chemotaxis Response of MDA-231 Cells Plated on 4 Extracellular Matrices				
	<u>Laminin</u>	<u>Type 1 Collagen</u>	<u>Type IV Collagen</u>	<u>Fibronectin</u>
EGF (30 ng/ml)	101 (0%)*	110 (5%)	108 (4%)	115 (8%)
FGF (30 ng/ml)	99 (0%)	102 (0%)	126 (24%)†	141 (38%)
PDGF (20 ng/ml)	109 (6%)	152 (49%)†	263 (141%)†	241 (130%)†

Chemotaxis Response of MCF-7 Cells Plated on Extracellular 4 Matrices				
	<u>Laminin</u>	<u>Type 1 Collagen</u>	<u>Type IV Collagen</u>	<u>Fibronectin</u>
EGF (30 ng/ml)	162 (0%)	150 (0%)	174 (2%)	169 (1%)
FGF (30 ng/ml)	155 (0%)	159 (0%)	163 (0%)	224 (10%)†
PDGF (20 ng/ml)	170 (0%)	324 (88%)†	395 (115%)†	415 (122%)†

Table I: % response compared to basal medium containing no growth factor. The data represent the mean of quadruplicate determinations.

† P < 0.05 compared to the control.

TABLE II

Chemokinetic Response to IGF-I of Each of the Four Cell Lines

	<u>T47D</u>	<u>BT-20</u>	<u>HS578T</u>	<u>ZR-75-1</u>
IGF-I (10 ng/ml)	697 \pm 41	397 \pm 51	563 \pm 70	699 \pm 80
IGF-I (30 ng/ml)	777 \pm 88	490 \pm 66	744 \pm 62	1005 \pm 99
IGF-I (100 ng/ml)	841 \pm 63	553 \pm 80	901 \pm 96	1353 \pm 105
Control	399 \pm 41	304 \pm 88	267 \pm 41	315 \pm 38

The results show the mean \pm one S.D. of quadruplicate experiments with 9-12 wounds per treatment per experiment.

TABLE III

Chemotaxis Response to IGF-I of Four Breast Cell Lines

	<u>T47D</u>	<u>BT-20</u>	<u>HS578T</u>	<u>ZR-75-1</u>
IGF-I (10 ng/ml)	226 (39%)	151 (11%)	244 (43%)	295 (59%)
IGF-I (30 ng/ml)	361 (76%)	195 (65%)	319 (61%)	399 (101%)
IGF-I (100 ng/ml)	369 (77%)	223 (88%)	366 (72%)	495 (181%)

The results represent the mean of quadruplicate determinations. The number in parenthesis is the cell number percentage increase over control media that contained 0.2% serum. All of the increases were significant ($P < 0.05$).

TABLE IV

Migration Responses of Breast Cell Lines Exposed to Anti-Integrin Antibodies

Cell Line	<u>T47D</u>	<u>BT-20</u>	<u>HS578T</u>	<u>ZR-75-1</u>
αv	-17%	0	-68%	-14%
$\alpha 5$	-81% +	-41%	---	-82%
$\beta 3$	-77% +	-62%	---	-50%
$\beta 5$	-11%	---	---	-41%
$\alpha 3$	---	-89%	-37%	-31%

The results are expressed as the percentage inhibition of the mean of 3 separate experiments. The percentage was calculated by dividing the number of cells migrating in the Boyden chamber assay by the number of cells migrating in response to 100 ng/ml of IGF-I in control cultures.

TABLE V

Chemokinetic Response of MCF-7 Cells to Estradiol and IGF-I

	<u>Number of migrating cells</u>	<u>% increase or decrease compared to control</u>
Control	370	---
IGF-I (10 ng/ml)	595	61%
IGF-I (30 ng/ml)	777	110%
Estradiol (10^{-7} M)	419	13%
IGF-I (30 ng/ml) + Estradiol (10^{-7} M)	701	189%
IGF-I (30 ng/ml) + Estradiol (10^{-6} M)	897	242%

The results represent the mean of three separate experiments measuring cell migration across a lateral surface in response to IGF-I or estradiol. 9-12 wounds were used per experiment.

TABLE VI

Chemokinetic Response of MDA-231 Cells to IGFBP-3 and IGFBP-5

	<u>Number of Migrating Cells</u>	<u>% Increase or Decrease in Migration</u>
Control	288	---
IGFBP-3 (100 ng/ml)	252	-13%
IGFBP-3 (500 ng/ml)	231	-20%
IGF-I (30 ng/ml)	462	61%
IGF-I (30 ng/ml) + IGFBP-3 (100 ng/ml)	360	25%
IGF-I (30 ng/ml) + IGFBP-3 (500 ng/ml)	276	-1%
IGFBP-5 (100 ng/ml)	299	0%
IGF-I (30 ng/ml)	489	70%
IGFBP-5 (500 ng/ml)	331	15%
IGF-I (30 ng/ml) + IGFBP-5 (100 ng/ml)	556	93%
IGF-I (30 ng/ml) + IGFBP-5 (500 ng/ml)	691	140%

The results represent the mean of three separate experiments. 9-12 wounds were used per data point.